



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷: C12N 15/00, A01K 67/027, C07K 14/56, C12N 15/86, 7/04, 7/01, 15/21, G01N 33/53	A2	(11) International Publication Number: WO 00/11151 (43) International Publication Date: 2 March 2000 (02.03.00)
(21) International Application Number: PCT/US99/19393 (22) International Filing Date: 25 August 1999 (25.08.99) (30) Priority Data: 09/139,902 25 August 1998 (25.08.98) US (71) Applicants: UNIVERSITY OF GEORGIA RESEARCH FOUNDATION, INC. [US/US]; The Boyd Graduate Studies Research Center, Athens, GA 30602-7411 (US). AVIGENICS, INC. [US/US]; 845 Oak Grove Avenue #220, Menlo Park, CA 94025 (US). (72) Inventors: IVARIE, Robert; 54 Jackson Street, Watkinsville, GA 30677 (US). HARVEY, Alex, J.; 188 Sunset Drive, Athens, GA 30606 (US). MURPHY, George, F., Jr.; 1723 Santa Cruz Avenue, Menlo Park, CA 94025 (US). RAPP, Jeffrey, C.; 48 Third Street, Watkinsville, GA 30677 (US). (74) Agents: CHOW, Y., Ping et al.; Heller Ehrman White & McAuliffe, 525 University Avenue, Palo Alto, CA 94301-1900 (US).		(81) Designated States: AE, AL, AM, AT, AT (Utility model), AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, CZ (Utility model), DE, DE (Utility model), DK, DK (Utility model), DM, EE, EE (Utility model), ES, FI, FI (Utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK (Utility model), SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>Without international search report and to be republished upon receipt of that report.</i>
(54) Title: DIRECT OVIDUCT TRANSGENESIS (57) Abstract Methods for preparing transgenic avians which express exogenous protein substantially only in their oviducts are disclosed. Each of the methods comprises delivering nucleic acid expression cassettes directly to the oviducts of the avians. The exogenous protein expressed by the expression cassette is secreted into the lumen of the avian oviduct and secreted into the eggs of the transgenic avians. Methods for preparing eggs which contain exogenous protein, such as human interferon, and methods for the production of proteins are also disclosed. The methods for direct oviduct transgenesis may also be used to assess the suitability of expression cassettes or exogenous proteins for expression in the avian oviduct.		

BEST AVAILABLE COPY

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

DIRECT OVIDUCT TRANSGENESIS

BACKGROUND OF THE INVENTION

5

a) Field of the Invention

This invention relates generally to methods of producing transgenic birds. In particular, the invention relates to methods of delivering a transgene directly to the avian oviduct to produce a bird which expresses an exogenous protein in its
10 avian oviduct and deposits that exogenous protein into its eggs.

a) Description of Related Art

Numerous natural and synthetic proteins are used in diagnostic and therapeutic applications; many others are in development or in clinical trials.
15 Current methods of protein production include isolation from natural sources and recombinant production in bacterial and mammalian cells. Because of the complexity and high cost of these methods of protein production, however, efforts are underway to develop alternatives. For example, methods for producing exogenous proteins in the milk of pigs, sheep, goats and cows have been reported.
20 These approaches suffer from several limitations, including long generation times between founder and production transgenic herds, extensive husbandry and veterinary costs, and variable levels of expression because of position effects at the site of the transgene insertion in the genome. Proteins are also being produced using milling and malting processes from barley and rye. However, plant post-
25 translational modifications differ from vertebrate post-translational modifications, which often has a critical effect on the biological activity of the exogenous proteins.

The avian reproductive system, including that of the chicken, is well described. The egg of the hen consists of several layers which are secreted upon
30 the yolk during its passage through the oviduct. The production of an egg begins

with formation of the large yolk in the ovary of the hen. The unfertilized oocyte is positioned on top of the yolk sac. Upon ovulation or release of the yolk from the ovary, it passes into the infundibulum of the oviduct where it is fertilized if sperm are present. It then moves into the magnum of the oviduct which is lined with
5 tubular gland cells. These cells secrete the egg-white proteins, including ovalbumin, lysozyme, ovomucoid, conalbumin and ovomucin, into the lumen of the magnum where they are deposited onto the avian embryo and yolk.

The hen oviduct is an excellent organ for a protein bioreactor because of high levels of protein production, proper folding and post-translation modification
10 of the target protein, and ease of product recovery. As a result, efforts have been made to create transgenic chickens expressing exogenous proteins in the oviduct (see PCT publication WO 97/47739). However, the production of transgenic chickens can be both time-consuming and inefficient. In fact, a key limitation of using any animal as a bioreactor is the time required (approximately 9 months for
15 chickens, 2-3 years for ungulates) to introduce the desired transgene into the animal's genome.

Because of the nature of the pharmaceutical and biotechnology industries, achieving gram quantities of protein production in 6 months or less is very desirable. A short turn-around time, for instance, would allow for rapid
20 optimization of transgene expression and protein secretion. It would also provide a supply of protein adequate for product processing studies and preclinical trials.

The hen offers a unique system for efficient direct transgenesis of the magnum gland, but initial attempts have yielded poor results. Plasmid DNAs carrying transgenes have been introduced directly into the magnum of mature hens
25 by electroporation (Ochiai et al., *Poultry Science*, 1998, 77:299-302). Due to the large size of the oviduct of mature hens, the transient persistence of the plasmid DNAs in the cells and the inefficiency of organ electroporation, only very low levels of protein were detected in the oviduct tissue of sacrificed hens and no expressed protein was reported as being detected in the egg. Other attempts have

involved the transfection of magnum cells with expression cassettes only after excision of the cells from the bird and preparation of an oviduct cell culture (Sanders et al., *Endocrinology*, 1985, 116:398-405; Sanders et al., *Biochemistry*, 1988, 27:6550-6557; Schweers et al., *Journal of Biological Chemistry*, 1990, 265:7590-7595; Otten et al., *Molecular Endocrinology*, 1988, 2:143-147; Schweers et al., *Journal of Biological Chemistry*, 1991, 266:10490-10497).

Thus, there exists a need for a rapid route to the expression of an exogenous protein in an avian oviduct and the resulting deposition of the protein in the avian egg.

10

SUMMARY OF THE INVENTION

The present invention provides methods for producing transgenic avians by direct oviduct transgenesis which satisfy the need for a rapid route to the expression of an exogenous protein in an avian oviduct and deposition of the expressed protein in the avian egg.

In one embodiment of the invention, a method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct is provided that comprises delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter.

In another embodiment of the invention, a method for preparing a transgenic avian which lays eggs containing exogenous protein is provided. The method of this embodiment comprises first preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct (as described in the previous paragraph) and then allowing the immature avian to grow to maturity,

so that the exogenous protein is secreted into the oviduct lumen of the mature avian and deposited into eggs laid by the avian.

In another embodiment of the invention, a method for preparing a transgenic avian which lays eggs containing exogenous protein is provided which comprises delivering a nucleic acid expression cassette directly to the oviduct of a mature avian, where the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter, and where the exogenous protein is expressed in the avian's oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

In an alternative embodiment of the invention, a method for producing an avian egg that contains exogenous protein is provided. The method of this embodiment comprises first delivering directly to the oviduct of an immature or mature avian a nucleic acid expression cassette which comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which is operably linked to the promoter and which codes for an exogenous protein, and then, if the avian is immature, allowing the avian to grow to maturity, wherein the exogenous protein is expressed in the oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

Still another embodiment of the invention provides a method for producing a protein. This method comprises producing an avian egg that contains exogenous protein as previously described and then isolating the exogenous protein from the egg.

A method for testing the efficiency of the expression of a transgene in an avian oviduct is also provided which comprises delivering a nucleic acid expression cassette directly to the oviduct of an avian, wherein the expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter, and assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.

In another embodiment of the invention, a method for testing the ability of a promoter to facilitate expression of a transgene in an avian oviduct is provided which comprises the following steps: delivering a nucleic acid expression cassette directly to the oviduct of an avian, where the nucleic acid expression cassette
5 comprises (i) the promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and assaying the lumen fluid of the oviduct for the amount or presence of the exogenous.

In another embodiment, the invention provides a method for screening a preparation of retroviral particles for deleterious mutations. The method of this
10 embodiment comprises first delivering the retroviral particles from a single preparation to the oviduct of an avian, wherein the retroviral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter. In the next step, the method comprises assaying for the presence or
15 amount of the exogenous protein in the fluid of the lumen of the oviduct.

In an alternative embodiment of the invention, a method for testing the suitability of a transgene for expression in an avian oviduct or for secretion of its expression product into the lumen of the oviduct and into the eggs of an avian is provided which comprises the following steps: delivering a nucleic acid
20 expression cassette directly to the oviduct of an immature avian, wherein the expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; allowing the immature avian to grow to maturity; and assaying for the amount or presence of the exogenous protein in the egg white of eggs laid by the
25 avian.

In further embodiments, the present invention provides for avians having a transgene encoding an exogenous protein substantially only in their oviducts, where the avians secrete into their eggs the protein expressed by the transgene. The invention also provides for avians having an exogenous protein substantially

only in their oviducts, where the avians secrete the exogenous protein into their eggs.

An intact avian egg containing protein exogenous to an avian egg is further provided by the present invention.

- 5 In still other embodiments of the invention, expression vectors and delivery vehicles particularly useful for the methods of the invention are provided. For instance, an avian leukosis virus pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus is provided. Additionally, an isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at
10 least 12 nucleotide-long fragment thereof, or a sequence that hybridizes thereto, wherein the polynucleotide is not a fragment of an interferon alpha-2b gene found in nature, is provided. Preferably, the fragments are at least about 18 nucleotides long. Most preferably, the fragments are at least about 60 nucleotides long. An expression vector comprising the polynucleotide sequence operably linked to a
15 promoter is also provided.

DETAILED DESCRIPTION OF THE INVENTION

20 a) Definitions and General Parameters

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

- The term "avian" means a bird of any known species or type. The term includes the various known strains of *Gallus gallus*, or chickens, (for example,
25 White Leghorn, Brown Leghorn, Barred-Rock, Sussex, New Hampshire, Rhode Island, Australorp, Minorca, Amro, California Gray, Italian Partridge-colored), as well as turkeys, pheasants, quails, duck, and other poultry commonly bred in commercial quantities.

"Direct delivery" refers to delivery which specifically and physically targets one cell type, tissue, compartment, or organ and avoids even transient exposure of other sites of the organism.

A "nucleic acid sequence" or "polynucleotide" includes, but is not limited to, eucaryotic mRNA, cDNA, genomic DNA, and synthetic DNA and RNA sequences, comprising the natural nucleoside bases adenine, guanine, cytosine, thymidine, and uracil. The term also encompasses sequences having one or more modified nucleosides. The terms "polynucleotide" and "oligonucleotide" are used interchangeably herein. No limitation as to length or to synthetic origin are suggested by the use of either of these terms herein.

A "coding sequence" or "open reading frame" is a polynucleotide or nucleic acid sequence which is transcribed (in the case of DNA) or translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence will usually be located 3' to the coding sequence.

Nucleic acid "control sequences" refers to translational start and stop codons, promoter sequences, ribosome binding sites, polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, as necessary and sufficient for the transcription and translation of a given coding sequence in a defined host cell. Examples of control sequences suitable for eucaryotic cells are promoters, polyadenylation signals, and enhancers. All of these control sequences need not be present in a recombinant vector, so long as those necessary and sufficient for the transcription and translation of the desired gene are present.

A "signal peptide" is responsible for transport of the protein out of the cell. In the majority of secreted proteins, the sequence is at the N-terminus of the nascent protein and is cleaved during synthesis and translocation into the

endoplasmic reticulum. Secretory DNA sequences in general are derived from genes encoding secreted proteins of the same species of the transgenic animal. Such secretory DNA sequences are preferably derived from genes encoding polypeptides secreted from the cell type targeted for tissue-specific expression.

5 Secretory DNA sequences, however, are not limited to such sequences.

“Operably or operatively linked” refers to the configuration of the coding and control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. A coding sequence is operably linked to or
10 under the control of transcriptional regulatory regions in a cell when DNA polymerase will bind the promoter sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet
15 transcribed sequences can be present between a promoter sequence and the coding sequence and the promoter sequence can still be considered “operably linked” to the coding sequence.

The term “exogenous protein” means a protein or polypeptide not naturally present in a particular tissue or cell, a protein that is the expression product of an
20 exogenous gene, an exogenous expression construct or a transgene, or a protein not naturally present in a given quantity in a particular tissue or cell.

The terms “heterologous” and “exogenous” as they relate to nucleic acid sequences such as coding sequences and control sequences, denote sequences that are not normally associated with a region of a recombinant construct, and/or are
25 not normally associated with a particular cell or tissue. Thus, a “heterologous” region of a nucleic acid construct is an identifiable segment of nucleic acid within or attached to another nucleic acid molecule that is not found in association with the other molecule in nature. For example, a heterologous region of a construct could include a coding sequence flanked by sequences not found in association

with the coding sequence in nature. Another example of a heterologous coding sequence is a construct where the coding sequence itself is not found in nature (e.g., synthetic sequences having codons different from the native gene).

Similarly, a host cell transformed with a construct which is not normally present in the host cell would be considered heterologous for purposes of this invention.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, naturally-occurring polynucleotides or polypeptides present in a living animal are not isolated, but the same polynucleotides or polypeptides could be part of a vector or composition, and be isolated in that such vector or composition is not part of its natural environment.

"Magnum" is that part of the oviduct between the infundibulum and the isthmus containing tubular gland cells that synthesize and secrete the egg white proteins of the egg.

A "magnum-specific" promoter is a promoter which is primarily or exclusively active in the tubular gland cells of the magnum.

The expression products described herein may consist of proteinaceous material having a defined chemical structure. However, the precise structure depends on a number of factors, particularly chemical modifications common to proteins. For example, since all proteins contain ionizable amino and carboxyl groups, the protein may be obtained in acidic or basic salt form, or in neutral form. The primary amino acid sequence may be derivatized using sugar molecules (glycosylation) or by other chemical derivatizations involving covalent or ionic attachment with, for example, lipids, phosphate, acetyl groups and the like, often occurring through association with saccharides. These modifications may occur *in vitro*, or *in vivo*, the latter being performed by a host cell through post-translational processing systems. Such modifications may increase or decrease the biological activity of the molecule, and such chemically modified molecules are also intended to come within the scope of the invention.

Alternative methods of cloning, amplification, expression, and purification will be apparent to the skilled artisan. Representative methods are disclosed in Sambrook, Fritsch, and Maniatis, *Molecular Cloning, a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory (1989).

5 “Vector” means a polynucleotide comprised of single strand, double strand, circular, or supercoiled DNA or RNA. A vector typically comprises some combination of the following elements operatively linked at appropriate distances for allowing functional gene expression: replication origin, promoter, enhancer, 5’ mRNA leader sequence, ribosomal binding site, coding sequence, termination and
10 polyadenylation sites, and selectable marker sequences. One or more of these elements may be omitted in specific applications.

In some embodiments the promoter will be modified by the addition or deletion of sequences, or replaced with alternative sequences, including natural and synthetic sequences as well as sequences which may be a combination of
15 synthetic and natural sequences. Many eukaryotic promoters contain two types of recognition sequences: the TATA box and the upstream promoter elements. The former, located upstream of the transcription initiation site, is involved in directing RNA polymerase to initiate transcription at the correct site, while the latter appears to determine the rate of transcription and is upstream of the TATA box.
20 Enhancer elements can also stimulate transcription from linked promoters, but many function exclusively in a particular cell type. Many enhancer/promoter elements derived from viruses, e.g. the SV40, the Rous sarcoma virus (RSV), and cytomegalovirus (CMV) promoters are active in a wide array of cell types. The nucleic acid sequence inserted in the cloning site may have any open reading
25 frame encoding a polypeptide of interest, with the proviso that where the coding sequence encodes a polypeptide of interest, it should lack cryptic splice sites which can block production of appropriate mRNA molecules and/or produce aberrantly spliced or abnormal mRNA molecules.

The termination region which is employed primarily will be one of convenience, since termination regions appear to be relatively interchangeable. The termination region may be native to the intended nucleic acid sequence of interest, or may be derived from another source.

5 A vector is preferably constructed so that the particular coding sequence is located in the vector with the appropriate regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control sequences. Modification of the sequences encoding the particular protein of
10 interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can
15 be cloned directly into an expression cassette which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

The terms "expression construct", "nucleic acid expression cassette" and "transgene" are used interchangeably herein. The terms refer to heterologous
20 polynucleotide sequences containing a desired coding sequence and control sequences in operable linkage, so that cells transformed with these sequences are capable of producing the encoded product. In order to effect transformation, the expression cassette may optionally be included on a discrete vector; however, the relevant polynucleotide may also be an integrative vector which has or can
25 become integrated into the host chromosome.

Suitable marker sequences for identification and isolation of correctly modified cells include green, yellow, and blue fluorescent proteins (GFP, YFP, and BFP, respectively). Other suitable markers include thymidine kinase (tk), dihydrofolate reductase (DHFR), and aminoglycoside phosphotransferase (APH).

The latter imparts resistance to the aminoglycoside antibiotics, such as kanamycin, neomycin, and geneticin. These, and other marker genes such as those encoding chloramphenicol acetyltransferase (CAT), β -lactamase (β -la), and β -galactosidase (β -gal), may be incorporated into the primary nucleic acid cassette along with the gene expressing the desired exogenous protein, or the selection markers may be
5 contained on separate vectors and cotransfected.

A "reporter gene" is a gene that "reports" its activity in a cell by the presence of the protein that it encodes.

A "plasmid" is a small, circular DNA vector capable of independent
10 replication within a bacterial or yeast host cell.

A "delivery vehicle" facilitates or helps facilitate the entry of a heterologous DNA or RNA sequence into a cell. The delivery vehicle may optionally be a virus which encapsulates a vector containing the expression cassette and aids its transfer into the cell. In some cases, the viral delivery vehicle
15 even integrates the transgene it carries into the genome of the host cell it infects. Alternatively, the delivery vehicle may be non-viral, such as a liposome.

A "regulatory element" is a DNA sequence contained within a gene to which a transcription factor(s) binds and alters the activity of the gene's promoter either positively (induction) or negatively (repression).

20 The terms "transformation", "transduction", and "transfection" all denote the introduction of a polynucleotide into a cell, such as an avian tubular gland cell.

The term "sequences which hybridize thereto" means polynucleotide sequences which are capable of forming Watson-Crick hydrogen bonds with another polynucleotide sequence under normal hybridization conditions, such as in
25 buffered (pH. 7.0-7.5) aqueous, saline solutions (for instance, 1 to 500 mM NaCl) at room temperature. Although normal hybridization conditions will depend on the length of the polynucleotides involved, typically they include the presence of at least one cation such as Na^+ , K^+ , Mg^{2+} , or Ca^{2+} , a near neutral pH, and temperatures less than 55°C. Although the sequences which hybridize to a

polynucleotide may be about 90%-100% complementary to the polynucleotide, if the sequences are of sufficient length, in solutions with high salt concentrations, and/or under low temperature conditions, polynucleotides with complementarity of 70% or above, or even just 50% or above, may hybridize to the polynucleotide.

- 5 Sequences which hybridize thereto typically comprise at least 12 nucleotides, and preferably at least about 15 nucleotides, which are complementary to the target polynucleotide.

b) Direct Oviduct Transgenesis

- 10 The present invention is directed to methods of producing transgenic birds and producing exogenous proteins which are deposited in the eggs of birds, each of which involves the delivery of an expression cassette directly to the oviduct of an avian, such as a chicken.

- One embodiment of the invention provides a method for preparing a
15 transgenic avian which expresses an exogenous protein substantially only in its oviduct. This method comprises delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is
20 operably linked to the promoter.

- Preferably, the avian of the method described in the previous paragraph is allowed to grow to maturity. The exogenous protein which is expressed in the oviduct is secreted into the oviduct lumen of the avian and is deposited into eggs laid by the avian. Thus, in another embodiment of the invention, a method for
25 preparing a transgenic avian which lays eggs containing exogenous protein is provided which comprises first preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct as described in the previous paragraph and then allowing the immature avian to grow to maturity.

In an alternative embodiment of the invention, a method of producing an avian egg which contains exogenous protein is provided. This method comprises delivering an expression cassette directly to the oviduct of an immature avian, where the expression cassette comprises the operably linked pair of (i) a promoter
5 which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein. This method requires the additional step of allowing the immature avian to mature. When the avian reaches the stage where it begins to lay eggs (maturity), the exogenous protein which is expressed in the avian oviduct and secreted into the lumen of the oviduct is deposited into the eggs laid by the
10 avian.

In another embodiment of the invention, a method for producing protein is provided which comprises first producing an avian egg that contains exogenous protein as described in the previous paragraph and then isolating the exogenous protein from the avian egg.

15 In all of the methods of the invention, the nucleic acid expression cassette is preferably delivered directly to the magnum cells of the immature oviduct.

The avian in each of the methods of the invention may optionally be selected from the group consisting of a chicken, turkey, goose, and quail.

In a preferred embodiment of each of the methods of the invention, the
20 immature avian is an immature chicken. An immature chicken is a chicken at a stage between hatch and the time when she begins to lay eggs (about 20 to about 24 weeks old). In a further preferred embodiment, the immature chicken is from about 10 to about 20 weeks old. At 10-12 weeks of age, the pullet oviduct weighs only 0.2 grams and the magnum consists of about 4×10^7 cells. By 20 weeks,
25 which is when most hens begin to lay eggs, the oviduct has matured into a 30-50 gram organ with a parallel increase in tubular gland cell number. By accessing the oviduct for transgenesis before it is mature, a higher percentage of cells can be modified, as compared to a mature hen oviduct.

In preferred embodiments of the methods of the invention, the immature avian is subjected to a steroid hormone treatment prior to delivery of the expression cassette. The steroid treatment comprises the administration of estrogen, progesterone, or testosterone, or combinations thereof. From hatch to 17 weeks, the cells of the magnum of chickens can be transiently induced to undergo a burst of proliferation by combined treatment with estrogen, progesterone, and/or testosterone (Yu & Marquardt, *Poultry Science*, 1973, 53:1096-1105; Brant & Nalbandov, *Poultry Science*, 1956, 35:692-700; Kohler et al., *J. Cell. Biol.*, 1969, 40:8-27; Oka and Schimke, *J. Cell Biol.*, 1969, 43:123-137; Palmiter and Wrenn, *J. Cell Biol.*, 1971, 50:598-615). Cessation of hormone treatment allows the magnum cells to resume their normal developmental program. In the present invention, the steroid treatment preferably comprises administration of estrogen, either alone or, preferably, in combination with progesterone or testosterone.

Estrogen alone can optionally be used alone either in the form of an implanted 13-15 mg diethylstilbestrol pellet or by injections of estrogen dissolved in a carrier. Injections of the hormone are preferred to implantation of a pellet because cessation of hormonal treatment is easier and two or more different hormones can be coinjected. Estrogen can be in the form of estrone, estradiol or diethylstilbestrol (DES); but DES is preferred because it is the most potent form. Doses can range from 0.1 mg to 24 mg per bird, depending on the weight of the bird. Typically doses for a 1 kg hen will be 1 mg of DES injected intramuscularly (typically the breast muscle), once per day, in a volume of 0.1 ml of 95% ethanol, Mazola oil or sesame oil.

While estrogen alone can induce proliferation of immature magnum cells, cotreatment with progesterone or testosterone greatly enhances the proliferative effect of estrogen. Therefore DES is preferably coinjected either with progesterone or testosterone, and most preferably with progesterone because testosterone is difficult to obtain due to its classification by the Drug Enforcement Agency as an anabolic steroid. For a 1 kg pullet (a young domestic hen), 1 to 3

mg of testosterone or 0.1 to 0.8 mg of progesterone should be coinjected along with 1.0 mg of DES. The preferred method is to dissolve 10 milligrams (mg) of DES and 8 mg of progesterone per milliliter (ml) of sesame oil. 0.1 ml doses are applied once a day by injection into the breast muscle.

- 5 In an alternative embodiment of the invention, an immature, non-chicken avian is subjected to a steroid hormone treatment prior to the delivery of the expression cassette. Estrogen and progesterone have been shown to have effects on the immature oviduct of Japanese Quails, ducks, and gulls that are similar to those in chicken (see Korpela et al., *Gen. Comp. Endocrinol.*, 1981, 44: 230-232).
- 10 One of ordinary skill in the art will be able to readily adapt dosage recommendations established for chickens to other types of birds.

For an avian of any type which is subjected to the methods of the invention, the hormone treatment is preferably administered daily and is optionally begun from about two to about ten days before introduction of the expression cassette

15 into the magnum. Either directly after transfection or transduction of the magnum or up to about 1 to 2 days later, the hormone administration is ceased, allowing the oviduct to develop normally.

In a preferred embodiment, the hormones are injected in the morning on days 1 through 3 (see dosages listed above). A procedure for directly accessing

20 the oviduct such as surgery or laparoscopy, including injection of the expression vector into the lumen of the magnum, is performed in the morning of day 4. Additional hormone injections are performed in the afternoon of day 4 and in the morning of day 5. The day 5 injection is optional. Depending on when the procedure is carried out, the treated hen will start to lay eggs containing the

25 desired protein within four to ten weeks.

Alternatively, all of the methods of the invention may comprise delivery of the nucleic acid expression cassette directly to the oviduct of a mature avian (an avian which is already laying eggs), instead of the preferred immature avian. For instance, an alternative method for preparing a transgenic avian which lays eggs

containing exogenous protein comprises delivering a nucleic acid expression cassette directly to the oviduct of a mature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which
5 is operably linked to the promoter, where the exogenous protein is expressed in the avian's oviduct, secreted into the lumen of the oviduct and deposited into eggs laid by the avian.

As a further example of how the methods of the invention may be adapted for use with mature avians, an alternative method for producing an avian egg that
10 contains exogenous protein, comprises delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which is operably linked to the promoter and which codes for an exogenous protein, where the exogenous protein is expressed
15 in the oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

In any of the methods of the invention, application of the expression cassette directly to the oviduct can be achieved by surgery. A surgical procedure involving an opening of the abdominal wall, is one option.

20 In a preferred embodiment, the surgical procedure used to access the avian oviduct comprises endoscopy of the abdominal cavity or laparoscopy. The laparoscope is used to visualize the oviduct while a fine gauge needle is inserted via a cannula and solution containing the expression cassette (optionally in a delivery vehicle such as a virus) is injected into the lumen of the magnum.
25 Laparoscopic grasping forceps or a suction clamp hooked to a vacuum source can be used to secure the oviduct.

In an alternative embodiment, a non-surgical method of directly accessing the oviduct for transgenesis may be used instead. One such suitable non-surgical method is transcloacal catheterization. Transcloacal catheterization comprises

first inserting a needle or tube (preferably a feeding needle) into the cloaca. The needle is then fed through the shell gland, isthmus, and into the lumen of the magnum. The solution is injected when the feeding needle reaches this position. The whole catheterization process may optionally be image-guided.

- 5 In an alternative embodiment of the invention, a method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct is provided which comprises delivering a nucleic acid expression cassette directly to the oviduct of an avian via laparoscopy or transcloacal catheterization, wherein the nucleic acid expression cassette comprises (i) a promoter which is
- 10 active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter.

- The expression cassette which is used in the methods of the invention must comprise the coding sequence of the exogenous protein of interest. The expression cassette must also contain a promoter which is active in cells of the
- 15 magnum and is operably linked with the nucleic acid sequence encoding the exogenous protein to be expressed. In one embodiment of the invention, a constitutive promoter, such as a cytomegalovirus (CMV), Rous sarcoma virus (RSV), or β -actin promoter is used. It is a distinct advantage of this method over traditional transgenic approaches that this method does not require tissue-specific
- 20 promoter elements. Alternatively, a magnum-specific promoter, such as the ovalbumin, conalbumin, ovomucoid, transferrin, or lysozyme promoter or a portion thereof, can instead be used in the methods of the present invention.

- The exogenous protein which is expressed in the avian oviduct preferably comprises a signal peptide sequence (encoded by the nucleic acid expression
- 25 cassette delivered to the avian oviduct) which facilitates secretion of the protein into the lumen of the oviduct and deposition of the protein into eggs laid by the avian. The protein which is expressed in the oviduct may be optionally expressed with its own native signal peptide that allows it to be efficiently secreted into the lumen of the oviduct and deposited into an egg. However, in other embodiments

the expression cassette may further comprise an operably linked sequence which codes for a signal peptide that is heterologous to the exogenous protein but which can still direct secretion of the exogenous protein into the lumen of the oviduct. A polynucleotide coding for an engineered signal peptide such as that occurring in egg white prelysozyme may be included in the expression cassette and operably linked to the coding sequence of the expression cassette (Jigami et al., *Gene*, 1986, 43:273-279). Such a signal peptide would preferably be expressed as a fusion protein with the exogenous protein of interest. The presence of the signal peptide allows secretion of the protein into the lumen of the magnum, and subsequently into the egg.

For purposes of application to the oviduct, the expression cassette is typically inserted within a larger vector to create an expression vector. The vector may optionally be an autonomously replicating vector. Alternatively, the vector may optionally be an integrative vector. In such an embodiment, the expression cassette is flanked by integration sequences. In addition to the coding sequence, the promoter and the optional secretory DNA sequence, the expression cassette or vector may also include assorted additional regulatory elements and control sequences such as enhancers. For instance, the SV40 enhancer is known to be useful in increasing expression from the ovalbumin promoter. Termination signals, splicing signals, and the like may also be included on the expression vector. A reporter or marker sequence may optionally be included on the expression vector as well.

Transfection of the magnum cells with plasmid DNAs containing the expression cassette can be performed using a chemical-mediated method or a ballistic-type delivery system, such as a gene-gun. Transfection of plasmid DNAs normally results in transient expression in the magnum as the plasmids are lost over time. Alternative embodiments offer the means of obtaining persistence of the plasmid's transgene within the oviduct. In one of these embodiments, a DNA replication sequence is included on the plasmid so that it will replicate stably and

persistently during magnum cell proliferation and differentiation. In another embodiment, the transgene is flanked with integration sequences and cotransfected with a plasmid encoding protein(s) capable of integrating the transgene into the chicken genome.

5 Preferably, the delivery of the expression cassette to the cells of the oviduct is aided by the use of a delivery vehicle. In a preferred embodiment of the present invention, the expression cassette is delivered directly to the oviduct within a delivery vehicle such as an adenovirus, a retrovirus, or a liposome. Alternatively, other types of viruses may be used as delivery vehicles.

10 To introduce transgenes directly into magnum cells, a variety of viral-mediated methods can be used. Human adenoviruses, such as those described in Fisher and Watanabe, *Cardiovascular Research*, 1996, 31:E86-95 and Lou et al., *Journal of Orthopedic Research*, 1996, 14:513-517, both herein incorporated by reference, have been shown to be efficient means of introducing genes into
15 chicken cells. Very high titers can be achieved (10^{10}), thus allowing a high multiplicity of infection (MOI). The human adenovirus vectors described above are replication-deficient and do not integrate into magnum cell DNA. Thus, the transgene persists only transiently in cells and long-term expression is not expected. Therefore, when a replication-defective adenovirus is used as a delivery
20 vehicle it is preferable to introduce the adenovirus vector into the magnum just before or during the onset of lay (20 weeks of age).

In another embodiment of the invention which can offer long-term expression, any retrovirus able to infect magnum cells may be used to introduce the transgene into magnum cells. Because retroviruses require rapidly dividing
25 cells to integrate efficiently into the host cell's DNA, use of the optional steroid hormone treatment on the immature avian prior to delivery of the nucleic acid cassette will help promote efficient transgenesis of the magnum cells. Therefore, when a retrovirus is used as a delivery vehicle for the expression cassette, prior

administration of the steroid hormone treatment to the immature chicken is strongly preferred.

For example, in preferred embodiments of the invention, a replication-deficient retrovirus derived from the avian leukosis virus (ALV) is used in the methods of the invention as a delivery vehicle for the expression cassette. An ALV-based retroviral vector has been used to produce germline transgenic Brown Leghorns (Cosset et al., *Journal of Virology*, 1991, 65:3388-94; Thoraval et al., *Transgenic Research*, 1995, 4:369-377). High titers can be achieved (5×10^6), but this virus requires that the target cell be dividing. The target magnum cells of an immature bird are undergoing rapid mitosis, especially after treatment of the bird with hormones. The ALV retroviral vector is replication-deficient. Thus, upon integration of the transgene into the chicken's genome, no virus will be produced during subsequent maturation of the oviduct and the egg laying lifetime of the hen.

In another embodiment of the methods of the invention, the vesicular stomatitis virus (VSV) pantropic retrovirus is used to deliver the expression cassette. The VSV pantropic vector, LZRN-L-G encodes β -galactosidase under control of the Moloney murine leukemia virus and is packaged with the G envelope glycoprotein (G-protein) of the vesicular stomatitis virus (VSV-G) (see Burns et al., *Proc. Natl. Acad. Sci. USA.*, 1993, 90:8033-8037). This transducing vector attaches directly to the outer phospholipid layer of cells in a receptor-independent manner. Hence, this transducing particle has been shown to integrate into the genomes of a variety of species, including chickens (Burns et al., *Proc. Natl. Acad. Sci. USA.*, 1993, 90:8033-8037; Lin et al., *Science*, 1994, 265:666-669; Yee et al., *Methods in Cell Biology*, 1994, 43:99-112). The VSV pantropic vector is replication-deficient. Thus, upon integration of the transgene into the chicken's genome, no virus will be produced during subsequent maturation of the oviduct and the egg laying lifetime of the hen.

The primary advantage of LZRN is the G-protein, which allows the vector particles to adsorb to tissues in a receptor-independent manner. This allows LZRN to transduce a wide range of tissues. However, LZRN is not an ideal vector for insertion of magnum expression cassettes due to poor cloning sites and limited room between the long terminal repeats (LTRs). The ALV-based vector, NLB, overcomes these deficiencies. However NLB is pseudotyped with an envelope protein that requires it to adsorb to cells via the cell-surface TV receptor. There are several ALV envelope proteins, each of which interacts with a specific TV receptor. For instance the envA envelope protein adsorbs to the TV-A receptor. The TV-A receptors, as well as other TV receptors are expressed on an unknown percentage of avian cells. For instance, almost all chicken fibroblast cells but only a small percentage of stage X embryo cells express the TV-A receptor. It is unknown whether chicken magnum cells express the TV-A receptor at levels adequate for transduction by ALV particles pseudotyped with the envA envelope protein. Therefore ALV particles alone may not be able to enter into magnum cells.

One aspect of the present invention provides for an ALV particle in which the G protein is inserted into the envelope of the ALV particle. This allows production of NLB vector packaged in a particle able to transduce cells directly through the phospholipid membrane, bypassing the requirement for cell-surface TV receptors. As an example of how an ALV-based retrovirus may be pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus, the cells which package the NLB vector encoding a CMV promoted interferon (IFN) gene are Isolde cells (which produce the gag, pol and envA proteins and are referred to as Isolde-NLB-CMV-IFN). The particles produced by this cell line are pseudotyped with the envA receptor, which specifically adsorbs to the TV-A receptor. Isolde-NLB-CMV-IFN cells are plated out at $3-5 \times 10^6$ cells per T75 tissue culture flask. The next day, they are transfected with an expression plasmid that expresses the G protein under control of the CMV promoter (pCMV-G) using

standard transfection protocols. In the following days, the transfected cells will produce NLB-CMV-IFN particles, of which a significant fraction are now pseudotyped with the G-protein. See the Example 3, below for a working example of a pseudotyping protocol. ALV particles pseudotyped with the G envelope glycoprotein have been found to successfully transduce the magnum cells of the chicken oviduct (see Example 4, below), whereas attempts to transduce magnum cells with ALV particles pseudotyped with the envA protein have not been successful.

It will be apparent to one of ordinary skill in the art that retroviruses other than the ones specifically mentioned here will be useful in the direct oviduct transgenesis methods described herein. Both replication-defective and replication-competent retroviruses can be used for the present invention, although replication-defective retroviruses are preferred.

Alternatively, instead of directly injecting a virus that comprises the expression cassette into the magnum in the methods of the invention, one injects the cells which produce the virus (helper cells). The helper cells can live for several days inside the magnum. During that time, the helper cells continuously secrete virus, thereby increasing the dosage and duration of the infection of the magnum. Any helper cell known to those skilled in the art to be compatible with the viral delivery vehicle chosen for use in the methods of the invention would be suitable for this application.

For instance, in one particular embodiment, a helper cell line that is producing high titers of ALV-based retrovirus is transfected with a vector encoding the VSV-G protein. The following day the helper cells will secrete ALV-based retroviruses containing the VSV-G protein inserted into the envelope. The cells are harvested in a manner that maintains viability and are typically immediately injected into the magnum's lumen.

Liposomes provide still another option for a delivery vehicle useful in the methods of the present invention. Liposomes increase intracellular stability and

increase uptake efficiency. Liposomes are hollow spherical vesicles composed of lipids arranged in a similar fashion as those lipids which make up the cell membrane. The appropriate use of liposomes as delivery vehicles in the present invention would be known to one of ordinary skill in the art. Numerous lipids are commercially available, and some, such as Lipofectin™, have been used to deliver genes into a variety of organs.

The delivery of the expression vector directly to the magnum portion of the oviduct can optionally be further aided by positioning an expression-cassette releasing entity directly in or adjacent to the magnum. The expression-cassette releasing entity can be a packet or suppository which releases plasmid DNA, liposomes, or viruses carrying the expression cassette into the magnum over some period of time. Alternatively, as discussed above with respect to retroviruses, the deposited expression-cassette releasing entity is a helper cell which both produces and secretes viral delivery vehicles into the oviduct.

As previously stated, the magnum can be treated at almost any time during development but certain times are particularly advantageous. At 10 to 15 weeks, the magnum of an avian such as a chicken is small and more readily saturated by virus or another carrier of an expression cassette. If retroviral delivery is used, direct oviduct transgenesis at this early stage may be preferred. In the case of retrovirus treatment, primordial magnum cells that contain the integrated transgene will amplify the transgene copy number (relative to the magnum) as the cells proliferate. After 15 weeks, the magnum of an avian such as a chicken is larger, requiring larger amounts of virus to facilitate efficient transgenesis. However, for transient transgenesis with adenovirus infection, liposome-mediated transfection, or plasmid-transfection, 15 to 20 weeks should be the optimal time period for treatment.

If a mature magnum is to be targeted, instead of the preferred immature magnum, then non-retroviral delivery methods such as liposome delivery are most suitable.

If a retrovirus is used as a delivery vehicle, it is especially advantageous to administer the aforementioned steroid hormone treatment to the immature avian prior to infection with the virus due to the resulting stimulation in growth and DNA synthesis. In other embodiments of the invention, however, alternative methods of stimulating magnum cell growth and DNA synthesis may be employed either alone or in conjunction with the aforementioned steroid hormone treatment. These modifications are likely to increase the integration efficiency of the retroviral vector used. One such alternative method involves the inclusion of an oncogene or growth factor gene in the genome of the retroviral vector.

Alternatively, when the magnum cells are infected with the retrovirus carrying the expression cassette, they may be coinfectd with a second virus which expresses an oncogene or growth factor. This second virus could be an adenovirus, which would offer the advantage of only transiently stimulating DNA synthesis, thereby not causing any detrimental effects to the bird.

Oncogenes/growth factors useful in the present invention include src, large T antigen of polyoma/SV-40 virus, E2F1, avian c-erb B, c-myc, and c-jun. These factors may optionally be coexpressed with an inhibitor of apoptosis (programmed cell death) such as bcl-2 or baculovirus p35. Over-expression of many oncogenes and growth factors can result in apoptosis, which is readily offset by expression of an apoptosis inhibitor such as bcl-2 or p35.

Use of the invention thus allows for targeted expression of exogenous proteins in oviduct cells with secretion into the lumen of the oviduct magnum and, ultimately, deposition in eggs whites. The exogenous protein may be packaged in the eggs in quantities of up to one gram or more per egg. The invention can be used to express, in large yields and at low cost, a wide range of desired proteins including those used as human and animal pharmaceuticals, diagnostics, and livestock feed additives. In preferred embodiments of the invention, the proteins which are expressed in the avian oviducts are human proteins. Proteins such as human growth hormone, interferon (such as interferon α , β , γ , or ω), lysozyme, and

β -casein are examples of proteins which are desirably expressed in the oviduct and deposited in eggs according to the methods of the invention. Other possible proteins to be expressed (or overexpressed) in the avian oviduct and isolated from eggs according to the methods of the invention include, but are not limited to,

5 albumin, α -1 antitrypsin, antithrombin III, collagen, factors VIII, IX, X (and the like), fibrinogen, hyaluronic acid, insulin, lactoferrin, protein C, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tissue-type plasminogen activator (tPA), feed additive enzymes, somatotropin, and chymotrypsin. Genetically engineered

10 antibodies, such as immunotoxins which bind to surface antigens on human tumor cells and destroy them, can also be expressed for use as pharmaceuticals or diagnostics. In one preferred embodiment, the expression cassettes used in the methods of the invention code for human interferon alpha-2b.

Proteins which are isolated from avian eggs produced by the methods of the

15 invention are provided by additional aspects of the invention. For instance, an interferon, such as human interferon alpha-2b, which is isolated from eggs produced according to the methods of the invention is provided by the present invention.

In preferred embodiments of the methods of the invention, the sequence on

20 the expression cassette which codes for an exogenous protein not normally expressed in an avian, or at least not in an avian oviduct, has been modified to use codons which are commonly used in genes expressed by the avian and particularly genes expressed in the avian oviduct. One such coding sequence useful for expression in the chicken oviduct is the sequence shown in SEQ ID NO:1. This

25 sequence codes for pre-human interferon alpha-2b, but contains a number of altered codons to more closely match the codon usage preferences demonstrated by genes normally expressed in the chicken oviduct. Thus, an alternative aspect of the invention provides an isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at least 12 nucleotide-long

fragment thereof, or a sequence that hybridizes thereto, wherein the polynucleotide is not a fragment of an interferon alpha-2b gene found in nature. Optionally, the signal peptide of the pre-human interferon alpha-2b coding sequence may be replaced with a heterologous signal peptide, such as that from lysozyme, for use in any of the methods of the invention. Expression vectors comprising this polynucleotide and a promoter which is operably linked to the polynucleotide are also provided by the present invention.

In another embodiment, the invention provides an avian having a transgene encoding an exogenous protein substantially only in its oviduct, where the avian secretes the exogenous protein into its eggs. In another embodiment, the invention provides an avian having an exogenous protein substantially only in its oviduct, where the avian secretes the exogenous protein into its eggs. Furthermore, the invention provides an avian which lays eggs containing exogenous proteins such as human proteins. Optionally, the exogenous protein which the avian secretes into its egg comprises a human interferon.

In still another embodiment, the present invention provides an intact avian egg that contains protein exogenous to the avian egg. Optionally, the exogenous protein contained in the egg comprises a human interferon, such as human interferons alpha-2b.

To demonstrate the potential of direct oviduct transgenesis (DOT), hormone-induced magnums of pullets were transduced with retroviral particles carrying the human interferon alpha-2b gene (IFN) under control of the CMV promoter (see the specific examples, Examples 1-4, below). The construction of the vector used for these experiments is outlined in the specific example, Example 1, below. The production of the retroviral cell lines and particles are detailed in the specific examples, Examples 2 and 3, below. The retroviral particles used for the working example of direct oviduct transgenesis were pseudotyped with VSV-G. One week after the DOT operation, lumen fluid was sampled from two hens (Example 4, below) and compared to supernatants from excised oviducts as

negative and positive controls (Example 5, below). Both DOT-treated hens had significant levels of human interferon in the lumen fluid (Example 4, Table 1, below). Eggs from the same birds were collected and assayed for the presence of interferon in the egg white (Example 4, Table 2, below).

5 In addition to the usefulness of direct oviduct transgenesis for preparing transgenic avians, expressing transgenes in the avian oviduct, preparing eggs containing exogenous protein, and producing proteins, direct oviduct transgenesis provides a quick route to assessing the usefulness of a particular genetic construct or part of a genetic construct for expression in the avian oviduct. For instance, the efficiency of promoters, such as magnum-specific promoters, or other regulatory
10 elements may be rapidly tested. Alternatively, the coding sequence itself may be tested. For instance, the effectiveness of implementing codon changes within the coding sequence for expression in the magnum may be tested. Signal sequence effectiveness may also be tested using the methods of the invention. The tested
15 expression constructs, if satisfactory, can then optionally be used to generate germline transgenic avians where expression in the oviduct is desired. For instance, the tested expression cassettes may be delivered via a retrovirus to a developing avian embryo in an egg (see co-pending US patent application no. 09/173,864, filed October 16, 1998, and Bosselman et al., US Patent No. 5,162,
20 215).

Although the following methods, like all the methods of the invention, are preferably carried out using an immature avian, each of the methods can alternatively be performed using a mature avian. (If a mature avian is used, one of ordinary skill in the art will recognize that any steps directed to allowing the bird
25 to grow to maturity are no longer necessary.)

One embodiment of the invention provides a method for testing the efficiency of the expression of a transgene in an avian oviduct which comprises first delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a

promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter, and then assaying for the presence or amount of the exogenous protein in the lumen of the oviduct. In a preferred embodiment, the nucleic acid expression cassette is in a retroviral vector.

5 In another embodiment of the invention, a method for testing the ability of a promoter to facilitate expression of a transgene in an avian oviduct is provided which comprises the following steps: delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) the promoter and (ii) a nucleic acid sequence which codes
10 for an exogenous protein and which is operably linked to the promoter; and assaying for the presence or amount of the exogenous protein in the fluid of the lumen of the oviduct. In a preferred embodiment, the promoter is a magnum-specific promoter.

Typically, transgenes can be quickly tested for expression in the magnum
15 by sampling lumen fluid after the operation or other delivery of the nucleic acid expression cassette to the oviduct. Preferably, the lumen fluid is sampled at least about one week after delivery of the nucleic acid expression cassette to the oviduct and before the avian begins to lay eggs. Most preferably, the lumen fluid is sampled within about three to about six weeks after the operation (or other form of
20 delivery of the nucleic acid expression cassette). An example of such a procedure is illustrated in the specific example, Example 4, below.

The method by which the sample of lumen fluid is assayed will be dependent upon the identity of the exogenous protein. One of ordinary skill in the art will readily be able to identify a suitable method of establishing the presence
25 and/or amount of the exogenous protein within the fluid sample. In many instances, standard assays using antibodies or labeled substrates may be used.

The methods of the invention are especially useful for testing magnum-specific promoters in live birds. If the promoter of the expression cassette is magnum-specific, a hormone treatment is preferably administered to the immature

avian for at least about six to ten days after delivery of the nucleic acid expression cassette. If the preferred hormone treatment has been applied to the immature avian as previously described prior to delivery of the nucleic acid expression cassette to the oviduct, then the hormone treatment is preferably extended for
5 about 6 to about 10 days past the operation to ensure that the magnum is capable of expressing the magnum-specific promoters. Significant expression of magnum-specific genes is not detected in pullets unless hormone treatment is continued for at least 10 days (Brant and Nalbandov, 1956; O' Malley et al., 1979, *Recent Prog. Horm. Res.*, 35:1-46; Oka and Schimke, 1969; Yu and Marquardt, 1973, *Comp.*
10 *Biochem. Physiol. [B]*, 44:769-77; Yu and Marquardt, 1973, *Endocrinology*, 92:563-72). At about six to about ten days post-operation, the lumen fluid may be sampled and assayed for the presence of the desired secreted protein. In this way, transgenes with a magnum-specific promoter can be tested for activity.

A particular advantage of the methods of the invention is that they allow
15 the rapid testing of magnum-specific promoters in retroviral expression vectors. Currently it is not possible to transduce magnum cells with retroviral vectors once they have been excised from the pullet or hen and cultured via the oviduct explant assay (OEA) (Sanders and McKnight, 1985, *Endocrinology*, 116:398-405; Sanders and McKnight, 1988, *Biochemistry*, 27:6550-7). Although it is unknown
20 what is the block to transduction in the oviduct explant assay, it may be that the cells in the oviduct explant assay cease to divide and efficient transduction requires concomitant cellular proliferation. With direct oviduct transgenesis, the functionality of a magnum-specific promoter (from the *ovalbumin*, *lysozyme*, *conalbumin*, *ovomucoid* or *transferrin* genes, for example) in a preparation of
25 transduction particles could be quickly confirmed. This would allow the screening of particle preparations for deleterious mutations that arose during the production of the particles. Particle preparations that demonstrate activity in the magnum after direct oviduct transgenesis would then be used to produce a flock of transgenic chickens.

Similarly, a method for testing the suitability of a transgene for expression in an avian oviduct and/or for secretion of its expression product into the lumen of the oviduct and into the eggs of an avian is also provided which comprises the following steps: delivering a nucleic acid expression cassette directly to the

5 oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; allowing the immature avian to grow to maturity; and assaying for the amount or presence of the exogenous protein in the egg white of eggs laid by the avian. The method may

10 optionally further comprise the additional step of assaying the exogenous protein found in the lumen or in the egg for bioactivity. Preferably, but not necessarily, the protein in the egg is assayed after isolation from the egg.

Direct oviduct transgenesis can also be used to confirm that a given protein can be expressed in the magnum of live pullets and secreted into the egg white of

15 laying hens. A protein which is produced in this manner can then optionally be assayed to confirm bioactivity. Also, if the protein is present in the eggs in reasonably large quantities, the protein may be isolated and used for additional studies such as protein purification trials or efficacy studies.

In another embodiment of the invention, a method for screening a

20 preparation of viral particles for deleterious mutations is provided which comprises the following steps: delivering the viral particles from a single preparation to the oviduct of an immature avian, wherein the viral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is

25 operably linked to the promoter; and assaying fluid of the lumen of the oviduct for the amount of the exogenous protein which has been both expressed in the oviduct and secreted into the lumen of the oviduct. In a preferred embodiment, the viral particles comprise retroviral particles. In another preferred embodiment, the promoter of the viral particles' expression cassette is a magnum-specific promoter.

In another embodiment, the present invention provides a method for investigating the nature of an exogenous protein which has been expressed in an avian's oviduct and secreted into the egg white of the avian's eggs. The method of this embodiment comprises the following steps: delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the
5 nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; allowing the immature avian to grow to maturity; and assaying the exogenous protein found in the eggs laid by the avian for a particular
10 characteristic. In a preferred embodiment, the method further comprises the additional step of isolating the exogenous protein from the eggs laid by the immature avian prior to assaying the exogenous protein for the particular characteristic. The preferred characteristic may optionally be bioactivity or a structural characteristic. For instance, the nature of post-translational
15 modifications on the exogenous protein which has been expressed in the avian oviduct may be investigated.

c) Examples

The following specific examples are intended to illustrate the invention and
20 should not be construed as limiting the scope of the claims:

Example 1. Vector Construction.

To allow for the efficient replacement of the *lacZ* gene of pNLB with any transgene, an intermediate adaptor plasmid was first created, pNLB-Adapter.
25 pNLB-Adapter was created by inserting the chewed back *ApaI/ApaI* fragment of pNLB (Cosset et al., *J. Virol.*, 1991, 65:3388-94) into the chewed-back *KpnI/SacI* sites of pBluescriptKS(-). (In pNLB, the 5' *ApaI* resides 289 bp upstream of *lacZ*, and the 3' *ApaI* resides 3' of the 3' LTR and Gag segments). The filled-in *ClaI/XbaI* fragment of pCMV-IFN-MM (Moore et al., *Anal. Biochem.*, 1997,

247:203-209) was inserted into the chewed-back *KpnI/NdeI* sites of pNLB-Adapter, replacing *lacZ* with the CMV promoter and the *IFN-MM* gene and thereby creating pNLB-Adapter-CMV-IFN-MM. (In pNLB, *KpnI* resides 67 bp upstream of *lacZ* and *NdeI* resides 100 bp upstream of the *lacZ* stop codon.) The
5 *IFN-MM* gene (SEQ ID NO:1) codes for human interferon-alpha-2B (IFN). To create pNLB-CMV-IFN-MM, the *HindIII/BlpI* insert of pNLB (containing *lacZ*) was replaced with the *HindIII/BlpI* insert of pNLB-Adapter-CMV-IFN-MM. This two step cloning was necessary because direct ligation of blunt-ended fragments into the *HindIII/BlpI* sites of pNLB yielded mostly rearranged subclones, for
10 unknown reasons. A portion of the pNLB-CMV-IFN-MM vector sequence containing both the CMV promoter and *IFN-MM* is shown as SEQ ID NO:2.

Example 2. Production of a NLB-CMV-IFN-MM producing helper cell line.

Sentas and Isoldes were cultured in F10 (Gibco), 5% newborn calf serum
15 (Gibco), 1% chicken serum (Gibco), 50 microgram/milliliter (ml) phleomycin (Cayla Laboratories) and 50 microgram/ml hygromycin (Sigma cat. # H-3274). Transduction particles were produced as described in Cosset et al., *Virology*, 1993, 65:2288-94, with the noted exceptions. Two days after transfection of the retroviral vector into 9×10^5 Sentas, virus was harvested in fresh media for 6-16
20 hours and filtered. All of the media was used to transduce 3×10^6 Isoldes in 3 100 mm plates with polybrene added to a final concentration of 4 micrograms/ml. The following day the media was replaced with media containing 50 microgram/ml phleomycin, 50 microgram/ml hygromycin and 200 microgram/ml G418 (neomycin) (Sigma cat. # G-9516). After 10-12 days, single G418^R colonies were
25 isolated and transferred to 24-well plates. After 7-10 days, titers from each colony was determined by transduction of Sentas followed by G418 selection. Typically 2 out of 60 colonies gave titers at $1-3 \times 10^5$. Those colonies were expanded. The integrity of the CMV-IFN-MM expression cassette was confirmed by assaying for

interferon in the media of quail or chicken cells transduced with NLB-CMV-IFN-MM transduction particles.

Example 3. Production and concentration of VSV-G typed NLB-CMV-IFN-MM particles.

The retroviral particles were pseudotyped with the VSV-G envelope protein which enables receptor-independent adsorption of the particles (Burns et al., *Proc. Natl. Acad. Sci. USA*, 1993, 90:8033-7; Yee et al., *Methods Cell Biol.*, 1994, 43 Pt A:99-112). (Certain retroviral particles which were not pseudotyped with VSV-G had previously failed to lead to protein expression in the lumen through direct oviduct transgenesis.)

4.5×10^6 Isolde helper cells which produce NLB-CMV-IFN-MM transduction particles (Isolde-NLB-CMV-IFN-MM) were plated in 2 to 6 75 cm² tissue culture treated polystyrene flasks (typically 6) (Falcon catalog # 3084) with complete F10 with hygromycin, phleomycin and neomycin added. (Typically 3 confluent 25 cm² flasks were harvested). 4×10^5 Isolde-NLB-CMV-IFN-MM were plated per well in 1 6-well plate (Falcon catalog # 3046).

The next day, the 75 cm² flasks were transfected by mixing 40 micrograms of cesium chloride (CsCl) gradient-purified or Qiagen column-purified pCMV-G plasmid DNA and 3 mls of Optimem (Gibco-BRL) in a polystyrene tube and 46 microliters of DMRIE-C (Gibco-BRL) and 3 mls of Optimem in a second polystyrene tube. The plasmid pCMV-G expresses the G envelope glycoprotein of the vesicular stomatitis virus under control of the (CMV) promoter. The mixtures were combined, mixed and incubated for 15 minutes at room temperature. Meanwhile the flasks were washed twice with 12 mls of Optimem per wash. The six ml transfection mix was added to the flask followed by slow rocking for 4 hours in the 37°C CO₂ incubator. The mix was then aspirated, washed once with 12 mls of Optimem and refed with 20 mls complete F10 but with hygromycin and neomycin added (no phleomycin).

Typically two wells of the 6-well plate were transfected with pCMV-EGFP which expresses Enhanced Green Fluorescent Protein (EGFP; Clontech) under control of the CMV promoter. This served as a control for transfection efficiency.

At the same time as the 75 cm² flask transfection was being carried out, 1 or 2 wells of the 6-well plate were transfected by mixing 5 micrograms of purified pCMV-EGFP plasmid DNA (CsCl-purified or Qiagen-purified) and 0.25 mls of Optimem (Gibco-BRL) in a first polystyrene tube and 6 microliters of DMRIE-C (Gibco-BRL) and 0.25 mls of Optimem in a second polystyrene tube. The mixtures were combined, mixed and incubated for 15 minutes at room

temperature. Meanwhile the wells were washed twice with 2 mls of Optimem per wash. The 0.5 ml transfection mix was added to the flask followed by slow rocking for 4 hours in the 37°C CO₂ incubator. Next, the mix was aspirated, washed once with 2 mls of Optimem and refed with 2 mls complete F10, but with hygromycin and neomycin added (no phleomycin).

24 hours later the 6-well plate was checked for transfection efficiency. (Transfection efficiency (with pCMV-EGFP) should be at least 30% and can be as high as 80% with DMRIE-C.)

24 hours post-transfection, the medium was aspirated from the 75 cm² flasks and the flasks were washed 2X with 4 mls F10 with no serum added. 12 mls of F10 with 1% newborn calf serum (Gibco), 0.2% chicken serum and no drugs was added. 24 more hours later the medium was removed for filtration and new low serum F10 was added to the flasks. The harvested medium was filtered through a 0.45 micron filter (Gelman Sciences Acrodisc HT Tuffryn syringe filter, 0.45 micron) into a polypropylene tube (or a polyallomer quick-seal centrifuge tube, see below) and stored at 4°C until concentration. (Polystyrene tubes should not be used to store VSV-G typed particles. Polypropylene should be used instead.)

Particle supernatants were harvested from the flasks every 24 hours. VSV-G typed particle production peaked at 48-72 hours post-transfection (30-50% of

all particles were typed with VSV-G) and then trailed off to low levels by 120 hours (5-15% of all particles were typed with VSV-G).

At 120-144 hours post-transfection, the filtered supernatants were concentrated 10-100 fold as described in Allilioli et al., *Dev. Biol.*, 1994, 165:30-7, with the differences noted here. Harvested media was filtered by passage through a 0.45 μ m filter via a 60 ml syringe with an 18G needle attached to the filter, straight into 1 X 3 1/2 inch polyallomer quick-seal centrifuge tubes (Beckman, 342414). The tubes were topped off with incomplete F10, capped with UV sterilized caps and sealed. Tubes were spun in a Beckman 60Ti rotor precooled to 4°C at 37,400 rpm (RCF = 98,977 g) for 30 minutes at high acceleration and deceleration. After the spin, some fluid was drained with a syringe and the top removed with a razor blade. All but 0.4 to 1.2 mls of the supernatant was removed without disturbing the pellet. The tube was then placed in a 37°C incubator so that the pellet was immersed and vigorously rocked for one hour to resuspend the pellet. With a micropipettor, the pellet was further triturated by pipetting up and down several times. Aliquots of the concentrated particles were stored at -70°C. (Typically, a loss of 50 % of the live virus occurred.)

Particles were titered on both Sentas and Isoldes to determine the titer of envA-typed particles and VSV-G typed particles. 2-3 x10⁵ Isoldes and 2-3 x10⁵ Sentas were plated per well in separate 6-well plates with no drugs added. One or two days later, an aliquot of the concentrated particles was thawed and diluted 1 in 500 with F10, complete. Each well was replaced with 2 mls of F10, complete, and 4 microliters of 1.0 mg/ml polybrene. 2 to 20 microliters of diluted particles were added to both a Senta well and an Isolde well. Twenty four hours later, the media was replaced with F10, complete, plus 200 microgram/ml G418 (neomycin). This media was changed every two days until distinct colonies formed. The number of colonies in a Senta well gave the titer, after correction for dilution factors, of both envA-typed particles and VSV-G typed particles. The number of colonies in an Isolde well gave the titer, after correction for dilution factors, of VSV-G typed

particles. It is assumed that most VSV-G typed particles also contained the envA-encoded envelope proteins and thus adsorbed to Sentas via the tv-a receptor or directly through the phospholipid membrane. Since Isoldes produced the envA-encoded envelope proteins, adsorption by envA-typed particles was blocked.

- 5 Therefore, particles adsorbed to Isoldes only if the particles contain the VSV-G envelope protein.

Example 4. Direct oviduct transgenesis (DOT) by surgical procedure

White Leghorn pullets which were 11 weeks old were used in the example.

- 10 Pullets which are between 10 and 20 weeks old can be used in the procedure. One to ten days prior to treatment, the pullets are preferably given daily dosages of diethylstilbestrol (DES, a potent form of estrogen) and/or progesterone and/or testosterone to stimulate proliferation of magnum cells. Doses will range from 0.1 mg to 24 mg per bird, depending of the weight of the bird. Typically, doses for a
- 15 1 kg hen are 1 mg of DES and 0.8 mg of progesterone, injected intramuscularly in a volume of 0.1 ml of 95% ethanol or sesame oil. Testosterone may be substituted for progesterone in some experiments. Additional hormone injections may be given the day of surgery and for several days after. The day before treatment, the pullets are taken off of their diet. In this example, 1 mg of DES and 0.8 mg of
- 20 progesterone per kg of pullet was injected daily for three days.

- On the morning of the fourth day, the magnum of the oviduct was accessed by surgical procedures. Pullets were anesthetized with a standard dose of isoflurane. Aliquots of the concentrated particles were thawed on ice. The magnum region of the oviduct was approached through a left lateral abdominal
- 25 incision. Laparoscopic grasping forceps were used to secure the oviduct during the injection. 0.5-0.6 mls of particles (1.6×10^5 to 5×10^5 VSV-G typed particles from Example 3) were injected in two or three locations into the lumen of the magnum using a 1 ml syringe and 22G needle. The incision was sutured and the birds allowed to wake. The pullets were returned to their cages that afternoon

and given one last injection of DES and progesterone. Particle solutions remaining after injection were retitered on Isoldes and Sentas to confirm the viral titer. Six days later the same pullets were taken off their diet.

The next day the magnum was accessed through the same incision used for
5 the injections. 0.5 ml of phosphate-buffered saline (PBS) was injected into the lumen. The lumen was gently massaged to mix the PBS with the lumen fluid. The sample was then removed with the same needle/syringe and placed on ice. After surgery the pullets were returned to their cages.

The 0.1 ml samples removed from the lumen of DOT-treated hens one
10 week after transduction were assayed with an interferon-alpha-2B ELISA kit (Research Diagnostics, Inc.) using the high sensitivity protocol. The results are shown below in Table 1. Both DOT-treated hens had significant levels of human interferon in the lumen fluid. 0.1 ml samples from the media of chicken tubular gland cells two days after transfection with the indicated plasmids using the
15 oviduct explant assay protocol (OEA) were also assayed (see Example 5, below).

Table 1. Expression of interferon in the lumen of DOT-treated hens one week after treatment.

Protocol	Sample	pg/ml
DOT	Pullet # 4039 lumen sample	137.1
DOT	Pullet # 4041 lumen sample	189.7
OEA	pCMV-EGFP transfected TGC cells (<i>in vitro</i>).	2.9
OEA	pCMV-IFN-MM transfected TGC cells (<i>in vitro</i>).	1108.8

Twelve weeks later, eggs were collected from the laying hens and assayed for the presence of interferon in the egg white. Egg white samples were removed
20 from the eggs of untreated hens (treatment: "none") or from eggs of two hens that underwent direct oviduct transgenesis 1-2 weeks after those hens started to lay. 0.1 ml of egg white was assayed with an interferon-alpha-2B ELISA kit (Research

Diagnostics, Inc.) using the high sensitivity protocol. The amounts of interferon found in the eggs is shown in Table 2, below. One hen (#4039) had significant levels of interferon in the egg white as compared to egg white from untreated hens. The second hen (#4041), which did have interferon in the lumen one week after the DOT operation, did not have detectable levels of interferon in the white of her eggs. It is possible that the magnum cells of hen #4039 were transduced such that they remained in an active state after the magnum fully developed whereas the cells of hen #4041's magnum were sloughed off or silenced during development.

10

Table 2. Deposition of interferon in the egg white of DOT-treated hens.

Treatment	Date egg laid	Band #	pg/ml (ELISA)	pg/ml (Bioassay)
None	2/14/99	2369	31.2	0
None	2/11/99	1422	32.1	0
DOT with viral particles.	3/9/99	4039	179.0	291
DOT with viral particles.	3/10/99	4039	208.1	291
DOT with viral particles.	3/9/99	4041	32.2	ND
DOT with viral particles.	3/10/99	4041	23.1	ND

DOT: direct oviduct transgenesis; ND: not determined.

Bioactivity of human interferon in the egg white was determined by incubating human A-549 cells with dilutions of the egg white samples, challenging the cells with Menogvirus and counting viral-resistant plaques as described in Lewis, "Biological Assays for Interferons", in *Lymphokines and Interferons*, Clemens, ed., Oxford: IRL Press, pp.73-87, 1987. The interferon from hen #4039 was fully bioactive as determined by an anti-viral bioassay, confirming the expression of interferon in the hen's magnum.

15

Example 5. Oviduct explant assay (OEA).

The oviduct explant assay was performed as described in Sanders and McKnight, *Endocrinology*, 1985, 116:398-405, and Sanders and McKnight, *Biochemistry*, 1988, 27:6550-7, except that mature laying hens were used as the
5 source of magnum tissue and DMRIE-C was used instead of calcium phosphate for the precipitation method. Supernatants from cells transfected with the expression cassette pCMV-IFN-MM or pCMV-EGFP (see Example 3, above) were collected 48 hours post-transfection and assayed by ELISA. pCMV-IFN-MM was the same expression cassette that was inserted into pNLB-CMV-IFN-
10 MM (see Example 1, above) pCMV-EGFP served as a negative control plasmid. 0.1 ml of sample from the transfected cells was assayed with an interferon-alpha-2B ELISA kit (Research Diagnostics, Inc.) using the high sensitivity protocol. The results are shown in Table 1 above.

15 All documents cited in the above specification are herein incorporated by reference. Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as
20 claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

We claim:

1. A method for preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct, comprising:

5 delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter.

10

2. A method for preparing a transgenic avian which lays eggs containing exogenous protein, comprising:

preparing a transgenic avian which expresses an exogenous protein substantially only in its oviduct according to the method of claim 1; and

15

allowing the immature avian to grow to maturity, wherein the exogenous protein is secreted into the oviduct lumen of the mature avian and deposited into eggs laid by the avian.

3. The method of claim 1, further comprising a first step of administering a
20 steroid hormone treatment to the immature avian.

4. The method of claim 1, wherein the step of delivering the expression cassette comprises a method selected from laparoscopy, transcloacal catheterization, and opening the abdominal wall.

25

5. The method of claim 1, wherein delivery of the expression cassette is aided by a delivery vehicle selected from the group consisting of an adenovirus, a retrovirus, and a liposome.

6. The method of claim 5, wherein the retrovirus is derived from the avian leukosis virus (ALV) or the vesicular stomatitis virus (VSV).

7. The method of claim 5, wherein the retrovirus is an ALV-based retrovirus which has been pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus.

8. The method of claim 5, wherein the retrovirus comprises an oncogene or growth factor gene in its genome.

10

9. The method of claim 8, further comprising coexpression of an inhibitor of apoptosis.

10. The method of claim 5, wherein the delivery vehicle comprises a retrovirus and the method further comprises the additional step of coinfecting the oviduct with a retrovirus that expresses an oncogene or growth factor.

15

11. The method of claim 1, wherein said delivery step comprises

(a) preparing helper cells which produce a virus carrying the expression cassette; and

20

(b) injecting the helper cells into the oviduct.

12. The method of claim 1, wherein the immature avian is an immature chicken, turkey, duck, goose, or quail.

25

13. The method of claim 12, wherein the immature avian is an immature chicken which is from about 10 to about 20 weeks old.

14. The method of claim 1, wherein the nucleic acid expression cassette codes for a protein selected from the group consisting of human growth hormone, interferon, β -casein, α -1 antitrypsin, antithrombin III, collagen, factors VIII, factor IX, factor X, fibrinogen, hyaluronic acid, insulin, lactoferrin, protein C, erythropoietin (EPO), granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), tissue-type plasminogen activator (tPA), feed additive enzymes, somatotropin, and chymotrypsin.

15. The method of claim 14 wherein the nucleic acid expression cassette codes for interferon α -2b.

16. A method for preparing a transgenic avian which lays eggs containing exogenous protein, comprising:

delivering a nucleic acid expression cassette directly to the oviduct of a mature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter,

wherein the exogenous protein is expressed in the avian's oviduct, secreted into the lumen of the oviduct and deposited into eggs laid by the avian.

17. A method for producing an avian egg that contains exogenous protein, comprising:

delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter which is active in the avian oviduct and (ii) a nucleic acid sequence which is operably linked to the promoter and which codes for an exogenous protein; and

allowing the immature avian to grow to maturity,

wherein said exogenous protein is expressed in the oviduct, secreted into the lumen of the oviduct, and deposited into eggs laid by the avian.

18. A method for producing a protein, comprising:

- 5 producing an avian egg that contains exogenous protein according to the method of claim 17; and
 isolating said exogenous protein from said egg.

19. A method for testing the efficiency of the expression of a transgene in an
10 avian oviduct, comprising:

- delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and
15 assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.

20. A method for testing the ability of a promoter to facilitate expression of a transgene in an avian oviduct, comprising:

- 20 delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) the promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and
 assaying for the presence or amount of the exogenous protein in the
25 fluid of the lumen of the oviduct.

21. A method for screening a preparation of viral particles for deleterious mutations, comprising:

delivering the viral particles from a single preparation to the oviduct of an immature avian, wherein the viral particles contain nucleic acid expression cassettes comprising (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter; and

assaying for the presence or amount of the exogenous protein in the lumen of the oviduct.

22. A method for testing the suitability of a transgene for expression in an avian oviduct or for secretion of its expression product into the lumen of the oviduct and into the eggs of an avian, comprising:

delivering a nucleic acid expression cassette directly to the oviduct of an immature avian, wherein the nucleic acid expression cassette comprises (i) a promoter and (ii) a nucleic acid sequence which codes for an exogenous protein and which is operably linked to the promoter;

allowing said immature avian to grow to maturity; and

assaying for the presence or amount of the exogenous protein in the egg white of eggs laid by the avian.

23. An avian having a transgene encoding an exogenous protein substantially only in its oviduct, wherein the avian secretes the exogenous protein expressed by the transgene into its eggs.

24. An avian having an exogenous protein substantially only in its oviduct, wherein the avian secretes the exogenous protein into its eggs.

25. The avian of claim 24, wherein said exogenous protein comprises human interferon.

26. An intact avian egg containing protein exogenous to the avian egg.
27. The egg of claim 26, wherein said protein comprises human interferon.
- 5 28. An avian leukosis virus pseudotyped with the G envelope glycoprotein of the vesicular stomatitis virus.
29. An isolated polynucleotide comprising the sequence set forth in SEQ ID NO:1, the complement thereof, an at least 12 nucleotide-long fragment thereof, or
10 a sequence that hybridizes thereto, wherein said polynucleotide is not a fragment of an interferon alpha-2b gene found in nature.
30. An expression vector comprising:
- (i) the polynucleotide of claim 29; and
 - 15 (ii) a promoter, wherein said promoter is operably linked to said polynucleotide.

SEQUENCE LISTING

<110> Ivarie, Robert
Harvey, Alex J.
Murphy Jr., George F.
Rapp, Jeffrey C.

<120> Direct Oviduct Transgenesis

<130> 24011-0003P1

<140>

<141>

<150> 09/139,902

<151> 1998-08-25

<160> 3

<170> PatentIn Ver. 2.0

<210> 1

<211> 567

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: pre-human
IFN-alpha-2b coding sequence modified with codons
for the chicken magnum

<400> 1

atggctttga ctttgcctt actggtggct ctctggtgc tgagctgcaa gaggcagctgc 60
tctgtgggct gcgatctgcc tcagaccac agcctgggca gcaggaggac cctgatgctg 120
ctggctcaga tgaggagaat cagcctgttt agctgcctga aggataggca cgattttggc 180
tttctcaag aggagtttgg caaccagttt cagaaggctg agaccatccc tgtgctgcac 240
gagatgatcc agcagatctt taacctgttt agcaccaagg atagcagcgc tgcttgggat 300
gagaccctgc tggataagtt ttacaccgag ctgtaccagc agctgaacga tctggaggct 360
tgcgtgatcc agggcgtggg cgtgaccgag acccctctga tgaaggagga tagcatcctg 420
gctgtgagga agtactttca gaggatcacc ctgtacctga aggagaagaa gtacagcccc 480
tgcgcttggg aagtcgtgag ggctgagatc atgaggagct ttagcctgag caccaacctg 540
caagagagct tgaggtctaa ggagtaa 567

<210> 2

<211> 4369
 <212> DNA
 <213> Artificial Sequence

<220>
 <221> promoter
 <222> (3105)..(3787)
 <223> CMV promoter

<220>
 <221> CDS
 <222> (3798)..(4364)

<220>
 <223> Description of Artificial Sequence: partial
 pNLB-CMV-IFN-MM vector sequence

<400> 2
 ctagagtcgg ggcggccggc cgcttcgagc agacatgata agatacattg atgagtttgg 60
 acaaacacaca actagaatgc agtgaaaaaa atgcttttatt tgtgaaattt gtgatgctat 120
 tgctttatatt gtaaccatta taagctgcaa taaacaagtt aacaacaaca attgcattca 180
 ttttatgttt cagggttcagg gggagggtgtg ggagggtttt taaagcaagt aaaacctcta 240
 caaatgtggt aaaatcgata aggatccgtc gaccgatgcc cttgagagcc ttcaaccacag 300
 tcagctcctt ccggtgggcg cggggcatga ctatcgctgc cgcacttatg actgtcttct 360
 ttatcatgca actcgtagga cagggtgccg cagcgctctt ccgcttcctc gctcactgac 420
 tcgctgcgct cggtcgcttc gctgcggcga gcggtatcag ctactcaaa ggcggtaata 480
 cggttatcca cagaatcagg ggataacgca ggaaagaaca tgtgagcaaa aggccagcaa 540
 aagccagga accgtaaaaa ggccgcgttg ctggcgtttt tccataggct ccgccccct 600
 gacgagcatc aaaaaaatcg acgctcaagt cagagggtggc gaaacccgac aggactataa 660
 agataccagg cgtttcccc cgggaagctcc ctctgctgct ctctgttcc gacctgccc 720
 cttaccgat acctgtccgc ctttctccct tcgggaagcg tggcgcttcc tcaatgctca 780
 cgctgtaggt atctcagttc ggtgtaggtc gttcgctcca agctgggctg tgtgcacgaa 840
 cccccgttc agcccgaccg ctgcgcctta tccggtaact atcgtcttga gtccaaccgc 900
 gtaagacacg acttatcgcc actggcagca gccactggta acaggattag cagagcgagg 960
 tatgtaggcg gtgctacaga gttcttgaag tggtagccta actacggcta cactagaagg 1020
 acagtatttg gtatctgcgc tctgctgaag ccagttacct tcggaaaaag agttggtagc 1080

tcttgatccg gcaaacaac caccgctggt agcgggtggt ttttggttg caagcagcag 1140
attacgcgca gaaaaaagg atctcaagaa gatccttga tcttttctac ggggtctgac 1200
gctcagtga acgaaaactc acgttaagggt attttgggtca tgagattatc aaaaaggatc 1260
ttcacctaga tctttttaa ttaaaaatga agttttaa caatctaaag tatatatgag 1320
taaacttggt ctgacagtta ccaatgctta atcagtggg cacctatctc agcgatctgt 1380
ctatttcgtt catccatagt tgccctgactc cccgtcgtgt agataactac gatacgggag 1440
ggcttaccat ctggcccag tgctgcaatg ataccgcgag acccacgctc accggctcca 1500
gatttatcag caataaacca gccagccgga agggccgagc gcagaagtgg tcttgcaact 1560
ttatccgcct ccattccagt tattaattgt tgccgggaag ctagagtaag tagttcgcca 1620
gttaatagtt tgcgcaacgt tgttgccatt gctacaggca tcgtggtgtc acgctcgtcg 1680
tttggtatgg cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatcccc 1740
atgttgtgca aaaaagcgg tagctccttc ggtcctccga tcgttggtcag aagtaagttg 1800
gccgcagtgt tatcactcat ggttatggca gactgcata attctcttac tgtcatgcca 1860
tccgtaagat gcttttctgt gactggtgag tactcaacca agtcattctg agaatagtgt 1920
atgcggcgac cgagttgctc ttgcccggcg tcaatacggg ataataccgc gccacatagc 1980
agaactttaa aagtgtcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc 2040
ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg caccacaactg atcttcagca 2100
tcttttactt tcaccagcgt ttctgggtga gaaaaacag gaaggcaaaa tgccgcaaaa 2160
aagggaataa gggcgacacg gaaatgttga atactcatac tcttctttt tcaatattat 2220
tgaagcattt atcaggggta ttgtctcatg agcggataca tatttgaatg tatttagaaa 2280
aataaacaaa taggggttcc gcgcacattt ccccgaaaag tgccacctga cgcgcctgt 2340
agcggcgcat taagcgcggc ggggtgtggtg gttacgcgca gcgtgaccgc tacacttgcc 2400
agcgccttag cgcgcctcc ttctgcttcc ttcccttctt ttctcgccac gttegccggc 2460
tttccccgtc aagctctaaa tcgggggctc cctttagggt tccgatttag tgctttacgg 2520
cacctcgacc caaaaaact tgattagggt gatgggtcac gtagtgggac atcgccctga 2580
tagacggttt ttccgctttt gacgttgagg tccacgttct ttaatagtg actcttgctc 2640
caaactggaa caacactcaa ccctatctcg gtctattctt ttgatttata agggattttg 2700

ccgatttcgg cctattgggt aaaaaatgag ctgatttaac aaaaatttaa cgcgaaat 2760
 aacaaaatat taacgtttac aatttcccat tcgccattca ggctgcgcaa ctgttgggaa 2820
 gggcgatcgg tgcgggctc ttcgctatta cgccagccca agctaccatg ataagtaagt 2880
 aatattaagg tacgggaggt acttgagcg gccgcaataa aatatcttta ttttcattac 2940
 atctgtgtgt tgggtttttg tgtgaatcga tagtactaac atacgctctc catcaaaaca 3000
 aaacgaaaca aaacaaacta gcaaaatagg ctgtccccag tgcaagtgc ggtgccagaa 3060
 catttctcta tcgataggta ccgagctctt acgctgtcta gcccgatgt acgggcccaga 3120
 tatacgctt gacattgatt attgactagt tattaatagt aatcaattac ggggtcatta 3180
 gttcatagcc catatatgga gttccgctt acataactta cggtaaatgg cccgcctggc 3240
 tgaccgcca acgacccccg cccattgacg tcaataatga cgtatgttcc catagtaacg 3300
 ccaataggga ctttccattg acgtcaatgg gtggactatt tacggtaaac tgcccacttg 3360
 gcagtacatc aagtgtatca tatgccagt acgcccccta ttgacgtcaa tgacggtaaa 3420
 tggcccgctt ggcattatgc ccagtacatg accttatggg actttcctac ttggcagtac 3480
 atctacgtat tagtcatcgc tattaccatg gtgatgcggt tttggcagta catcaatggg 3540
 cgtggatagc ggtttgactc acggggattt ccaagtctcc accccattga cgtcaatggg 3600
 agtttgtttt ggcacaaaaa tcaacgggac tttccaaaat gtcgtaacaa ctccgcccc 3660
 ttgacgcaaa tgggcggtag gcgtgtacgg tgggaggtct atataagcag agctctctgg 3720
 ctaactagag aaccactgc ttaactggct tatcgaaatt aatacgactc actataggga 3780
 gaccggaagc tttcacc atg gct ttg acc ttt gcc tta ctg gtg gct ctc 3830
 Met Ala Leu Thr Phe Ala Leu Leu Val Ala Leu
 1 5 10
 ctg gtg ctg agc tgc aag agc agc tgc tct gtg ggc tgc gat ctg cct 3878
 Leu Val Leu Ser Cys Lys Ser Ser Cys Ser Val Gly Cys Asp Leu Pro
 15 20 25
 cag acc cac agc ctg ggc agc agg agg acc ctg atg ctg ctg gct cag 3926
 Gln Thr His Ser Leu Gly Ser Arg Arg Thr Leu Met Leu Leu Ala Gln
 30 35 40
 atg agg aga atc agc ctg ttt agc tgc ctg aag gat agg cac gat ttt 3974
 Met Arg Arg Ile Ser Leu Phe Ser Cys Leu Lys Asp Arg His Asp Phe
 45 50 55
 ggc ttt cct caa gag gag ttt ggc aac cag ttt cag aag gct gag acc 4022
 Gly Phe Pro Gln Glu Glu Phe Gly Asn Gln Phe Gln Lys Ala Glu Thr

60	65	70	75	
atc cct gtg ctg cac gag atg atc cag cag atc ttt aac ctg ttt agc				4070
Ile Pro Val Leu His Glu Met Ile Gln Gln Ile Phe Asn Leu Phe Ser				
	80	85	90	
acc aag gat agc agc gct gct tgg gat gag acc ctg ctg gat aag ttt				4118
Thr Lys Asp Ser Ser Ala Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe				
	95	100	105	
tac acc gag ctg tac cag cag ctg aac gat ctg gag gct tgc gtg atc				4166
Tyr Thr Glu Leu Tyr Gln Gln Leu Asn Asp Leu Glu Ala Cys Val Ile				
	110	115	120	
cag ggc gtg ggc gtg acc gag acc cct ctg atg aag gag gat agc atc				4214
Gln Gly Val Gly Val Thr Glu Thr Pro Leu Met Lys Glu Asp Ser Ile				
	125	130	135	
ctg gct gtg agg aag tac ttt cag agg atc acc ctg tac ctg aag gag				4262
Leu Ala Val Arg Lys Tyr Phe Gln Arg Ile Thr Leu Tyr Leu Lys Glu				
	140	145	150	155
aag aag tac agc ccc tgc gct tgg gaa gtc gtg agg gct gag atc atg				4310
Lys Lys Tyr Ser Pro Cys Ala Trp Glu Val Val Arg Ala Glu Ile Met				
	160	165	170	
agg agc ttt agc ctg agc acc aac ctg caa gag agc ttg agg tct aag				4358
Arg Ser Phe Ser Leu Ser Thr Asn Leu Gln Glu Ser Leu Arg Ser Lys				
	175	180	185	
gag taa aaagt				4369
Glu				

<210> 3
 <211> 188
 <212> PRT
 <213> Homo sapiens

<400> 3
 Met Ala Leu Thr Phe Ala Leu Leu Val Ala Leu Leu Val Leu Ser Cys
 1 5 10 15
 Lys Ser Ser Cys Ser Val Gly Cys Asp Leu Pro Gln Thr His Ser Leu
 20 25 30
 Gly Ser Arg Arg Thr Leu Met Leu Leu Ala Gln Met Arg Arg Ile Ser
 35 40 45
 Leu Phe Ser Cys Leu Lys Asp Arg His Asp Phe Gly Phe Pro Gln Glu
 50 55 60
 Glu Phe Gly Asn Gln Phe Gln Lys Ala Glu Thr Ile Pro Val Leu His
 65 70 75 80

Glu Met Ile Gln Gln Ile Phe Asn Leu Phe Ser Thr Lys Asp Ser Ser
85 90 95

Ala Ala Trp Asp Glu Thr Leu Leu Asp Lys Phe Tyr Thr Glu Leu Tyr
100 105 110

Gln Gln Leu Asn Asp Leu Glu Ala Cys Val Ile Gln Gly Val Gly Val
115 120 125

Thr Glu Thr Pro Leu Met Lys Glu Asp Ser Ile Leu Ala Val Arg Lys
130 135 140

Tyr Phe Gln Arg Ile Thr Leu Tyr Leu Lys Glu Lys Lys Tyr Ser Pro
145 150 155 160

Cys Ala Trp Glu Val Val Arg Ala Glu Ile Met Arg Ser Phe Ser Leu
165 170 175

Ser Thr Asn Leu Gln Glu Ser Leu Arg Ser Lys Glu
180 185

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.